Available online at www.sciencedirect.com

ScienceDirect

Biochimica et Biophysica Acta 1773 (2007) 1398 – 1406

Biochimica et Biophysica Acta BBA

www.elsevier.com/locate/bbamcr

Identification and characterization of two putative nuclear localization signals (NLS) in the DNA-binding protein NUCKS

Kirsten Grundt [a], Ingvild V. Haga [a], Henrik S. Huitfeldt [b,c], Anne Carine Østvold [a,c,*]

[a] *Department of Biochemistry, Institute of Basic Medical Sciences, University of Oslo, P.O. Box 1112, Blindern, 0317 Oslo, Norway*
[b] *Laboratory for Toxicopathology, Institute of Pathology, Rikshospitalet University Hospital, N-0027 Oslo, Norway*
[c] *Center for Cellular Stress Responses, University of Oslo, Norway*

Received 18 September 2006; received in revised form 21 May 2007; accepted 24 May 2007
Available online 2 June 2007

Abstract

Immunofluorescence analyses show that the vertebrate specific and DNA-binding protein NUCKS is distributed throughout the cytoplasm in mitotic cells and targeted to the reforming nuclei in late telophase of the cell cycle. Computer analysis of the primary structure of NUCKS revealed the presence of two regions of highly charged, basic residues, which were identified as potential nuclear localization signals (NLSs). One of these signals (NLS1) is highly conserved between the species investigated, and fits to the description of being a classical bipartite NLS. The other amino acid motif (NLS2) is less conserved and does not constitute a classical bipartite NLS consensus sequence. We have shown that each of the two putative NLSs is capable of translocating green fluorescent protein (GFP) into the nucleus. The highly conserved NLS1 is monopartite, resembling the signals of c-Myc and RanBP3. Surprisingly, a natural occurring splice variant of NUCKS lacking 40 amino acids including NLS1, is not capable of translocating a corresponding NUCKS–GFP fusion protein into the nucleus, indicating that NLS1 is the main nuclear localization signal in NUCKS. This is also confirmed by site-directed mutagenesis of the full-length protein. By GFP-immunoprecipitation and GST-pull down experiments, we show that NUCKS binds to importin α3 and importin α5 *in vitro*, suggesting that the nuclear targeting of NUCKS follows a receptor-mediated and energy-dependent import mechanism.

Keywords: NUCKS; Nuclear transport; NLS; Importin; Cell cycle

1. Introduction

NUCKS (nuclear, casein kinase and cyclin-dependent kinase substrate) is a highly phosphorylated protein present in a number of vertebrate cell types and tissues [1,2]. Interestingly, the gene exhibits increased expression in several cancer tissues [3,4]. The protein is soluble in 5% perchloric acid and the amino acid composition of NUCKS resembles that of the HMGB family of proteins (high mobility group protein HMG1, 2) consisting of 48% charged residues [1]. The apparent molecular mass of NUCKS, when examined by SDS PAGE, is approximately 50 kDa. However, the molecular mass of purified NUCKS from rat liver, determined by mass spectroscopy, is 28.4 kDa including posttranslational modifications, indicating that NUCKS migrates anomalously in SDS-polyacrylamide gels [1]. In contrast to vertebrate HMGB proteins, NUCKS is phosphorylated on multiple sites *in vivo* [1,5]. The level of phosphorylation is highest in proliferating cells, and in proliferating human cells 23 *in vivo* phosphosites are characterized. Most of the sites are typical CK-2 and proline directed kinase phosphorylation sites. However, two DNA-activated protein kinase sites and two sites for tyrosine kinases are also reported in both human and mouse cells. (http://www.phosphosite.org/ParentProteinHomePage.jsp?proteinId=4128&organismId=5) [6]. It has been shown that NUCKS binds to DNA *in vitro* [7,8]. Mitotic phosphorylation of Ser181 by Cdk1 prevents binding of NUCKS to mitotic chromatin [7]. This is also reported for HMGN1 and HMGA1a/b, which is phosphorylated by PKC in mitotic cells [9,10]. Indirect immunofluorescence staining and transient

* Corresponding author. Department of Biochemistry, Institute of Basic Medical Sciences, University of Oslo, P.O. Box 1112, Blindern, 0317 Oslo, Norway. Tel.: +4722851097; fax: +4722851436.
E-mail address: annecari@medisin.uio.no (A.C. Østvold).

0167-4889/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2007.05.013

expression of NUCKS fused to GFP, show that NUCKS is localized in the nuclear compartment of both proliferating cells [1], and non-proliferating cells (unpublished results). It is therefore expected that NUCKS, which is synthesized in the M/G1 phase of the cell cycle [11], is actively transported into the nucleus.

All known transport between the nucleus and the cytoplasm occurs through nuclear pore complexes (NPCs), large proteinaceous channels embedded in the nuclear envelope. Macromolecules of up to 45 kDa may pass the NPC by passive diffusion, whereas larger molecules require specific nuclear localization signals (NLSs) to be targeted specifically to the nucleus [12]. However, it has been shown that even smaller proteins (<20–30 kDa) require NLSs for efficient nuclear translocation [13]. The best characterized nuclear transport process occurs via receptor recognition of classical NLSs within proteins targeted for nuclear import [14]. Proteins bearing classical NLSs are recognized in the cytoplasm by specific members of the importin superfamily, of which there are multiple α- and β-forms [15]. There are six human importin α proteins (α1,3,4,5,6 and 7) that fall into three subfamilies, P (α1), Q (α3 and α4) and S (α5, α6 and α7) [16]. Importin α proteins recognize and bind the NLS, while importin β translocates the trimeric complex through the nuclear pore. The delivery of cargo proteins and recycling of transport receptors occurs in an energy-dependent process [17]. A classical NLS consists of a short sequence enriched in basic amino acids that can be either monopartite or bipartite. The first group contains 3–5 basic amino acids with the weak consensus Lys–Arg/Lys–X–Arg/Lys, similar to the NLS of Simian virus 40 large T antigen (PKKKRKV) [18]. The bipartite group of NLSs consists of two small clusters of basic amino acids separated by about a dozen amino acid residues as that of nucleoplasmin (KRPAATKKAGQAKKKK) [19].

No NLS has yet been defined for NUCKS. Since the biological functions of NUCKS probably is connected to nuclear events, it is of interest to investigate the mechanism whereby NUCKS is transported into the nucleus. In this work we have characterized two putative NLSs and investigated whether each of the two independently is capable of mediating nuclear accumulation of GFP, which (due to its small size) is otherwise equally distributed in the cell [20]. By site-directed mutagenesis we investigated whether both the two putative signals are effective in directing the full-length protein to the nuclear compartment. We also addressed the question whether importin α/β heterodimers are responsible for the nuclear import of NUCKS.

2. Materials and methods

2.1. Antibodies, DNA constructs and peptides

Mouse anti-Lamin A/C was purchased from Santa Cruz, and mouse anti-nucleoporin p62 was from BD Transduction Laboratories. Mouse anti-GFP antibodies were purchased from Roche, Basel, Switzerland. Goat anti-rabbit IgG conjugated to Alexa Fluor 488 was purchased from Molecular Probes and donkey anti-mouse IgG conjugated to Cy3 was from Jackson laboratories. Donkey anti-rabbit IgG conjugated to peroxidase were purchased from Amersham Life Science. Antibodies against the C-terminal end of the NUCKS protein were raised in rabbits and affinity purified as described in [1]. Polyclonal antibodies against importin α1, 3 and 5 were kindly provided by Dr. Matthias Köhler, Medical Faculty of the Charité, The Franz Volhard Clinik, HELIOS Klinikum-Berlin and the Max Delbrück Center for Molecular Medicine. Vectors containing GST in fusion with importin α1/PTAC 58, importin α3/Qip 1 and importin α5/NPI-1 were kindly provided by Dr. Toshihiro Sekimoto, Department of Frontier Biosciences, Osaka University, Osaka, Japan.

2.2. Synchronization of HeLa S3 cells

The cells were grown in Eagle's minimum essential medium supplemented with 2 mM glutamine, 1% non essential amino acids, 10% fetal bovine serum, penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37 °C in a 5% CO_2 incubator. Synchronization of cells was achieved by single or double thymidine blocks as described in [21]. Mitotic cells were obtained by incubation for 24 h with 0.05 μg ml^{-1} colcemide.

2.3. Indirect immunofluorescence staining of cultured cells

Synchronized HeLa S3 cells grown in plastic dishes were fixed for 25 min in 4% paraformaldehyde, washed in PBS and permeabilized for 15 min in 0.2% Triton X-100 in PBS. The cells were washed twice in PBS and twice in distilled water and dried. The cells were incubated with affinity purified rabbit anti-NUCKS antibodies (1:500) and mouse anti-Lamin A/C antibodies (1:100) or mouse anti-nucleoporin p62 (1:100) over night at 4 °C, followed by extensive washing with PBS and incubation for 1 h in a mixture of Alexa 488 conjugated donkey anti-rabbit IgG antibodies (1:100) and Cy3 conjugated donkey anti-mouse IgG antibodies (1:200). Stained slides were examined in a Leitz DM RXE fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany) equipped with an F-view digital camera controlled by AnalySis 3.0 software (Soft Imaging System GmbH, Munster, Germany). A HCX Plan Apo 40×/0.85 objective was used for image acquisition.

2.4. Construction of GFP reporter fusions

A 752-bp human NUCKS cDNA containing the entire open reading frame including the stop codon was produced by PCR using Platinum Taq High fidelity polymerase (Invitrogen) and appropriate primers. Another PCR product was produced with the human splice variant (AY823399) as template. The PCR products were sub cloned into pcDNA3.1/NT-GFP-TOPO (Invitrogen) in frame with GFP. The same cDNAs without stop codon were sub cloned into pcDNA3.1/CT-GFP-TOPO (Invitrogen) in frame with GFP. Deletion and mutation constructs were generated by PCR using appropriate primers and sub cloned into pcDNA3.1/NT-GFP-TOPO or pcDNA3.1/CT-GFP-TOPO vectors respectively. All constructs were verified by DNA sequencing (Lark Technologies Inc., Essex CM226TA, United Kingdom).

Oligonucleotides for PCR amplification of full-length NUCKS:

5′-atgtcgcggcctgtcagaaatagg-3′,
5′-agaccatcatcacaaaaaatcctcc-3′
5′-gatcctccccagaaggggcttcatc-3′

Oligonucleotides for deletion mutagenesis:

5′-gctacagtgacgccaagtccagtg-3′
5′-tagtgggagggcccgaatctcttcc-3′
5′-ggcagcgatgaagatttcctaatgg-3′
5′-gaagctaaaaataagaggcgatct-3′
5′-ttaatcttccattaggaaatcttcatcgct-3′
5′-gagcttctcggggagatgatcgaat-3′
5′-gagcttcttggggagatgatcgaat-3′
5′-gtcgaattttcttagtgggagggcc-3′
5′-gtttcttagtgggagggcccgaatc-3′
5′-ttatcgaattttcttagtgggagcgcc-3′

2.5. Site-directed mutagenesis

Point mutations within the full length protein were generated using a PCR protocol (Quick change, Stratagene) with GFP-NUCKS as template and appropriate primers. The constructs were verified by DNA sequencing (Lark Technologies Inc., Essex CM226TA, United Kingdom). The primers employed were as follows:

5′-gattcgggcgctcccactaagaaaattcg-3′
5′-cgaattttcttagtgggagcgcccgaatc-3′
5′-cctcccactaagacaattcgatcatctccc-3′
5′-gggagatgatcgaattgtcttagtgggagg-3′
5′-cctcccactaagaaaattggatcatctccc-3′
5′-gggagatgatccaattttcttagtgggagg-3′
5′-cctcccactaacaaaattggatcatctccc-3′
5′-gggagatgatccaattttcttagtgggagg-3′

2.6. Expression of NUCKS–GFP fusion constructs in cultured cells

Human embryonic kidney cells (HEK 293) were grown in plastic dishes to 50–70% confluence in D-MEM supplemented with 10% fetal bovine serum, penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37 °C in a 5% CO_2 incubator. Transient transfection was conducted using LipofectAMINE 2000 (Life Technology) according to the manufacturer's method. The fluorescence became detectable less than 24 h after transfection. The cells were fixed for 25 min in 4% paraformaldehyde and washed in PBS. DNA was stained with 7-aminoactinomycin (25 μg ml^{-1}), and stained slides were examined in an Olympus BX60 microscope using a 100× objective and photographs taken with a JVC KY-F50E CCD camera and AnalySIS software (Soft Imaging System, Munster, Germany).

2.7. Immunoprecipitation and immunoblot analysis

HEK 293 cells were transiently transfected with constructs encoding GFP, NLS1-GFP and GFP-NLS2. The immunoprecipitation procedure was performed using the Protein G Immunoprecipitation kit (Roche). At 24 h after transfection the cells were harvested and resuspended in lysis buffer supplied with the kit. The cells were lysed for 30 min followed by centrifugation at 12,000×*g* for 30 min. The supernatants were incubated with 0.4 μg/μl mouse monoclonal anti-GFP antibodies for 4 h at 4 °C, followed by addition of 50 μl Protein G agarose. The incubation proceeded over night at 4 °C. The complexes were collected by centrifugation followed by extensive washing as described by the supplier. The immunoprecipitates were incubated with bacterially expressed GST-importins (α3 and α5) for 2 h at 4 °C. Following centrifugation, the complexes were washed ×5 in L-buffer (described under preparation of cell lysates) and dissolved in 25 μl 2× Laemmli sample buffer. Attached proteins were separated by SDS-PAGE (10% gel), blotted onto nitrocellulose and immunostained using standard procedures.

2.8. Recombinant expression of importin α proteins

pGEX plasmids containing the entire ORFs of importin α1, α3 and α5 were transfected into *E. coli* BL-21 cells and expressed as GST fusion proteins at 37 °C for 2 h under 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) induction. Bacteria were lysed in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 (L-buffer) with 5 mg ml^{-1} lysozyme (Sigma) and protease inhibitors (Complete, Roche, Basel, Switzerland) for 30 min at room temperature, briefly sonicated and clarified by Eppendorf centrifugation (13000 rpm, 15 min).

2.9. Preparation of cell lysates

Approximately 2×10^7 HeLa S3 cells were harvested, washed in PBS and resuspended in 1.0 ml L-buffer containing 0,1 mM NaF, 0,1 mM vanadate and 0,01 mM okadaic acid. The cells were frozen in −70 °C, thawed and rotated for 20 min on ice prior to sonication for 30 s. The supernatant was collected after centrifugation for 15 min at 15000 rpm.

2.10. GST-pull down experiments

GST fusion proteins prebound to glutathione-Sepharose 4 Fast Flow beads (Sigma), were incubated with 200 μl cell lysate and rotated at 4 °C for 2 h followed by extensive washing with L-buffer. Sepharose beads were dissolved in 25 μl 2× Laemmli sample buffer and boiled for 2 min prior to centrifugation. Attached proteins were separated by SDS-PAGE (15% gel), blotted onto nitrocellulose and immunostained using standard procedures. Rabbit anti-NUCKS were diluted

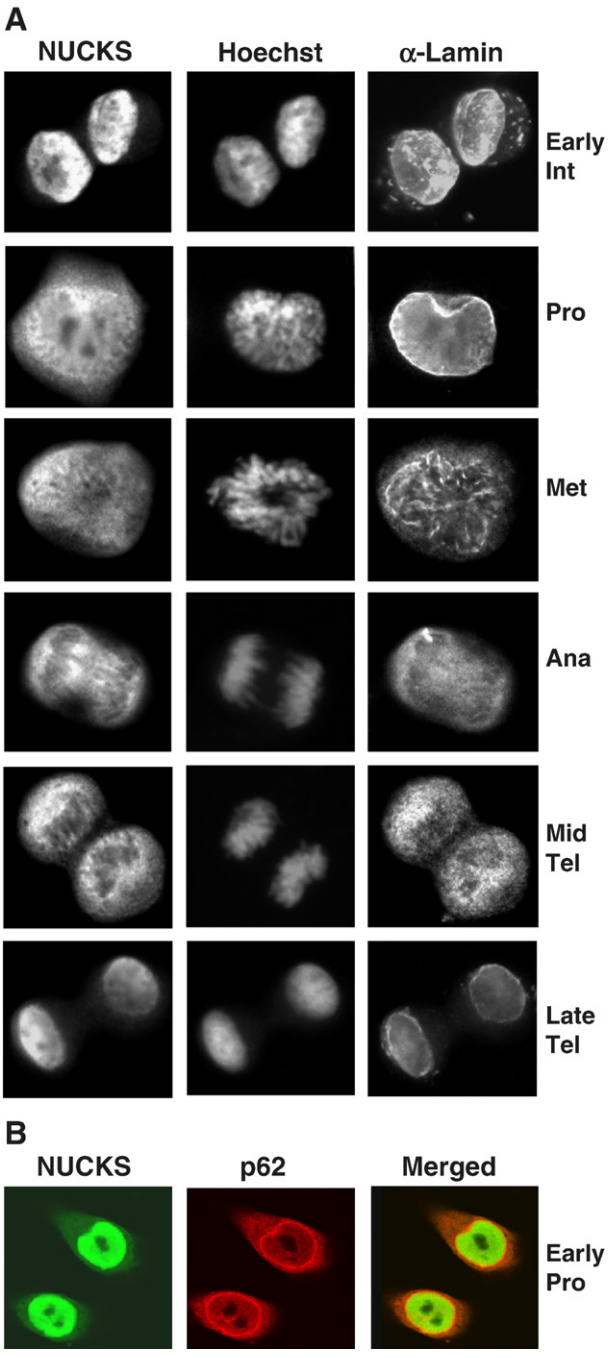

Fig. 1. Subcellular distribution of NUCKS. (A) Immunofluorescence analysis of NUCKS distribution in HeLa cells using affinity purified rabbit anti-NUCKS antibodies and mouse anti-lamin antibodies at indicated stages of the cell cycle. DNA was stained with Hoechst dye. (B) HeLa cells in early prophase were immunostained using rabbit anti-NUCKS and mouse anti-nucleoporin p62. Goat anti-rabbit IgG conjugated to Alexa 488 and donkey anti-mouse IgG conjugated to Cy3 were used as secondary antibodies.

1:4000, horseradish peroxidase-coupled secondary antibodies (1:10 000) and chemiluminescence reagents (Amersham) were used for detection.

3. Results

3.1. NUCKS is imported into the nuclei in late telophase of the cell cycle

It has been shown that NUCKS is located exclusively in the nuclei of cells in interphase of the cell cycle, but distributed to the cytoplasm in mitotic cells [7]. Furthermore, it has been reported that expression of NUCKS is cell cycle dependent, with highest expression in mitosis [11]. In order to more exactly determine at what phase of the cell cycle NUCKS is transported in and out of the nucleus, and whether the distribution in the cytoplasm in mitosis is due to disassembly of the nuclear envelope, HeLa cells were synchronized by a double thymidine block prior to harvesting the cells at different stages of the mitosis. The cells were fixed and stained with anti-NUCKS, anti-Lamin A/C and anti-nucleoporin p62 antibodies. As shown in Fig. 1A and B, NUCKS exhibits a cytoplasmic localization in early prophase of the cell cycle, concomitantly with release of nucleoporin p62 and breakdown of the pore complex, but before disassembly of the nuclear envelope. In early telophase, the NUCKS protein starts to accumulate in the newly formed nucleus, and in late telophase/early G1 the great majority of the protein is located in the nucleus (Fig. 1A).

3.2. NUCKS contains two NLSs capable of translocating GFP to the nucleus

We used a computer analysis program (pfscan at http://www.ensembl.org/Homo_sapiens/proview) to predict the probability of NLSs in human NUCKS. Two putative bipartite NLSs were identified. The first domain, NLS1, was predicted at residues $_{35}$–K–K–I–R–S–S–P–R–E–A–K–N–K–R–R–S–G–K–$_{52}$ and the second domain, NLS2, was predicted at residues $_{150}$–K–K–K–N–K–K–M–V–K–K–S–K–P–E–R–K–E–K–K–$_{168}$ (Fig. 2A). Fig. 2B shows a comparison between the sequences of the two putative NLSs found in human NUCKS and the sequences found in homologous proteins from chimpanzee, pig, chicken, frog and fish [22]. As seen from Fig. 2B, NLS1 is highly conserved among the species and corresponds to the classic consensus sequence [$(K/R)_2X_{10-12}$ $(K/R)_3$] of a bipartite NLS [23]. NLS2 on the other hand, is less conserved and is not strictly in agreement with the consensus sequence of a typical bipartite NLS.

In order to investigate whether each of the two putative NLS sequences is able to function independently in the nuclear import of GFP, various GFP–NUCKS fusion constructs were generated by deletion mutagenesis. NLS1–GFP contains 52 amino acids corresponding to the N-terminal end of NUCKS, C-terminally fused to GFP. GFP–NLS2 is another truncated form of NUCKS in which 130 amino acids of the N-terminal end are lacking. This construct contains both

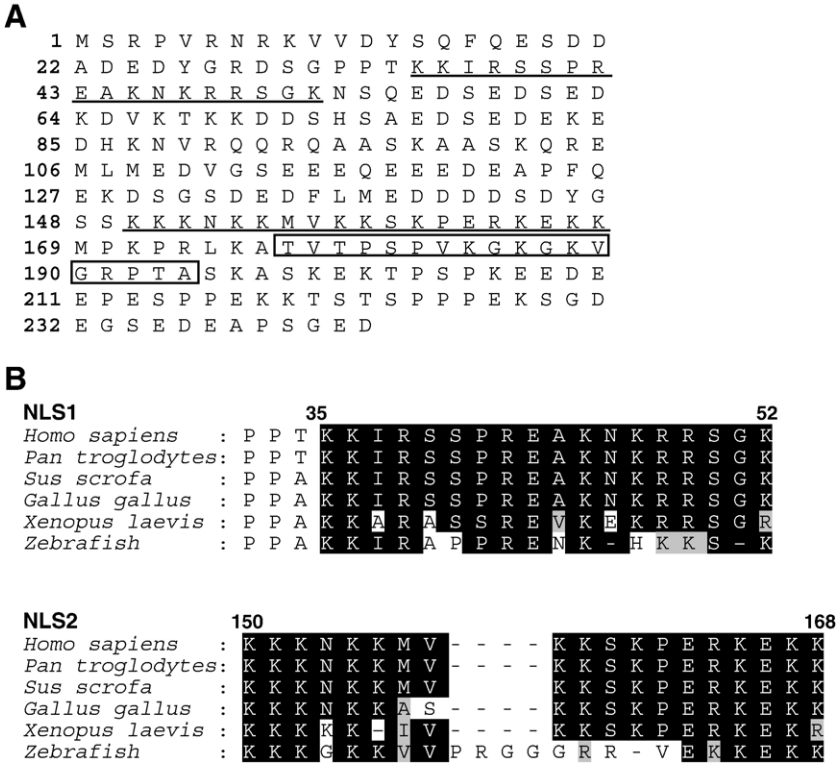

Fig. 2. The primary structure of human NUCKS. (A) Amino acid sequence of the NUCKS protein with two potential NLSs underlined. The DNA binding domain is boxed. (B) Comparison of the amino acid sequences of two putative NLSs in NUCKS from different species. Alignment of the amino acid sequence of human NUCKS with the amino acids sequences of putative homologous proteins from chimpanzee (Pan troglodytes, mRNA. XM_514141.1), pig (*Sus scrofa*, SsGI TC Report: TC221396), chicken (*Gallus gallus*, GgGI TC Report 44538), frog (*Xenopus laevis*, XGI TC Report: TC285445) and fish (Zebrafish, ZGI TC Report: TC139791). Identical residues with respect to the human sequence are shaded in black, conservative substitutions are shaded in grey.

NLS2 and the DNA-binding domain (DBD) and is N-terminally fused to GFP. HEK 293 cells were transiently transfected with the different deletion constructs and the expression of the GFP fusion proteins was visualized by direct fluorescence microscopy. As can be seen from Fig. 3A, NLS1–GFP was exclusively localized to the nucleus as compared to GFP alone, indicating that NLS1 directs nuclear import of GFP. When cells were transfected with NLS2, most of the fusion protein accumulated in the nuclei, but a faint fluorescence from the cytoplasm was also observed. Transfected cells from three different experiments were examined, and similar results were also obtained in transfected CHO cells (not shown). The results indicate that each of the two putative NLSs has the capacity to direct import of GFP to the nucleus. Surprisingly, when analysing a naturally occurring splice variant from human foetal brain lacking 40 amino acids (residue 14 to 54) including NLS1, the GFP fusion protein was localized in both the cytoplasm and the nucleus as is the case for GFP alone (Fig. 3B). This indicates that NLS2 is only capable of translocating the truncated C-terminal part of NUCKS into the nuclei.

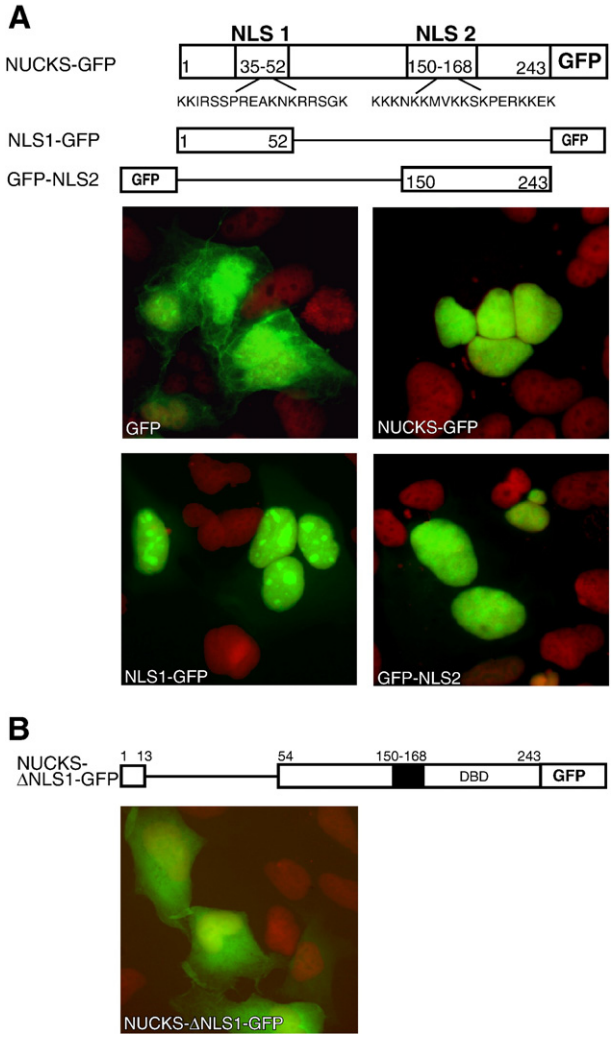

Fig. 3. Characterization of two nuclear localization signals in NUCKS by deletion mutagenesis. (A) The capability of the two putative NLSs to mediate nuclear transport was analysed by direct fluorescence microscopy. HEK 293 cells were transiently transfected with DNA constructs encoding: GFP, NUCKS–GFP, NLS1–GFP and GFP–NLS2. (B) A naturally occurring splice variant of NUCKS lacking 40 amino acids (residue 14 to 54) including NLS1 fused to GFP was transiently transfected into HEK 293 cells. At 24 h after transfection, living cells were fixed and counterstained with 7-aminoactinomycin (red). Merged red and green images are shown. Overview of the GFP-fusion constructs is shown in the upper panel.

3.3. Identification of NLS1 as a monopartite signal

To systematically define the region of NUCKS necessary for nuclear import, we performed further mutational analyses. Initially constructs containing each half of the putative bipartite motifs of NLS1 were made in fusion with GFP. In addition, two other truncated variants of GFP–NUCKS corresponding to 67 amino acids of the C-terminus containing the DBD but no NLS, and 34 amino acids of the N-terminal without NLS1, were also generated. The construct containing the N-terminal end of NLS1 was capable of translocating GFP to the nuclei, while the construct containing the C-terminal end was not (Fig. 4A), indicating that NLS1 is of a monopartite type despite the prediction of it being bipartite. The peptide corresponding to 34 amino acids of the N-terminal end of NUCKS was incapable of translocating GFP to the nucleus. Localization throughout the cell was also observed when cells were transfected with the GFP–DBD construct, implying that DNA binding alone cannot account for efficient nuclear localization (Fig. 4A). In the latter case, the green fluorescence observed in the nucleus is probably due to free diffusion of the GFP-fusion proteins (all fusions <45 kDa) as is observed with GFP alone.

NLS1 was mapped in greater detail by testing fusions containing point mutations and small deletions. The importance of arginine 42 in the N-terminal part of NLS1 was first investigated. When R42 was mutated to Q, the localization of GFP was predominantly nuclear, indicating that this arginine does not belong to the minimal NLS1 sequence (not shown). Furthermore, when the amino acids S39 to K52 was deleted, the localization of GFP was still nuclear, while the deletion mutant lacking I37 to K52 exhibited cytoplasmic localization of GFP (Fig. 4B), indicating that R38 is important. This was also the case when K35 was mutated to N (Fig. 4B). The basic core of the monopartite signal NLS1 is therefore $_{35}$KKIR$_{38}$, which resembles the NLS of cMyc (PAA**KRVK**LD) and NLS1 and NLS2 of the PSR (phosphatidylserine receptor) (KKIR, KKYR) [24,25]. Mutational analyses of the c-Myc sequence have revealed that the presence of a proline residue amino terminal to the basic residues is essential for efficient import of c-Myc to the nucleus [24]. We therefore mutated P32 to A in the NUCKS NLS1 sequence. When cells were transfected with this construct, the localization was both cytoplasmic and nuclear (Fig. 4B). The same point mutations in full-length NUCKS also impaired the nuclear accumulation of the protein compared to the wild type (Fig. 4C). All the experiments indicate that the minimal sequence in NLS1 necessary for efficient translocation of GFP fusion constructs into the nucleus is PPTKKIR.

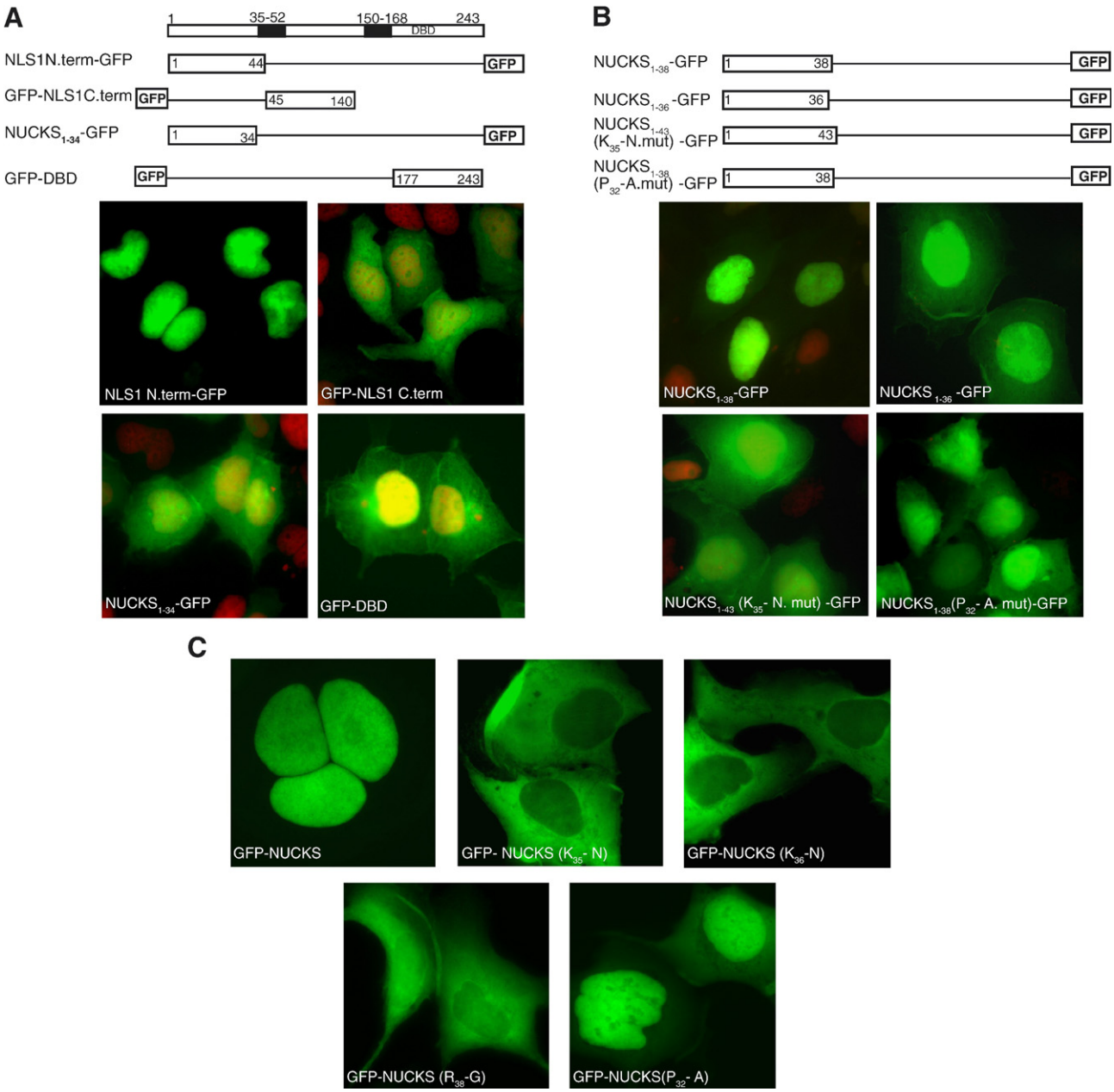

Fig. 4. Determination of NLS1 as monopartite by deletion mutagenesis and site directed mutagenesis. (A) To determine whether NLS1 is monopartite or bipartite, DNA constructs encoding truncated forms of NLS1 were made and analysed for the ability to target GFP to the nuclei. HEK 293 cells were transiently transfected with constructs encoding: $NLS1_{N.terminal}$ (C-terminally truncated)–GFP, GFP–$NLS1_{C.term}$ (N-terminally truncated), $NUCKS_{1-34}$–GFP (without NLS1) and GFP–DBD. (B) The essential amino acids of NLS1 were determined by mutational analysis. HEK 293 cells were transiently transfected with constructs encoding: NUCKS 1–38–GFP, NUCKS $_{1-36}$–GFP, NUCKS $_{1-43}$($K_{35} \rightarrow N$)–GFP and NUCKS $_{1-38}$($P_{32} \rightarrow A$)–GFP. Overview of the GFP-fusion constructs is shown in the upper panel. At 24 h after transfection, living cells were fixed, counterstained with 7-aminoactinomycin and analysed by direct immune fluorescence microscopy. Merged red and green images are shown. (C) The essential amino acids of NLS1 were determined by site directed mutagenesis of full-length NUCKS. HEK 293 cells were transiently transfected with constructs encoding GFP–NUCKS, GFP–NUCKS($K_{35} \rightarrow N$), GFP–NUCKS($K_{36} \rightarrow T$), GFPNUCKS($R_{38} \rightarrow G$) and GFP–NUCKS($P_{32} \rightarrow A$). At 24 h after transfection, living cells were fixed and analysed by fluorescence microscopy. Green images are shown.

To exclude the possibility that the position of GFP at the C-terminal or N-terminal end can influence the localization of the fusion protein, some of the constructs were made with GFP fused in the N-terminal end and the C-terminal end respectively. In all cases the position of GFP had no influence on the localization of the fusion protein (not shown).

3.4. NUCKS interacts preferentially with importin α3 and importin α5

Proteins containing canonical NLSs are recognized by a heterodimeric receptor consisting of importin-α/β, while a family of β-like proteins mediates import of karyophiles

bearing non classical NLSs [26–28]. Both RanBP3 NLS and the NLS of cMyc interact with importin α while the related NLS of the yeast protein Pho4p is translocated into the nucleus by Pse1p/Kap121p [29]. To investigate whether NUCKS is recognized by importin α, we performed *in vitro* GST-pull down experiments using purified GST-importins (importin α1, α3, α5), representing each of the subgroups of importin α molecules in mammalian cells. Importin α1, α3, and α5 were expressed in fusion with GST in bacteria, and the fusion products were isolated on glutathione-Sepharose beads. Following extensive washing, the beads were incubated with extracts from unsynchronized HeLa cells and extracts from HeLa cells arrested in metaphase of the cell cycle by colcemide. Since NUCKS is expressed in the M/G1 phase of the cell cycle

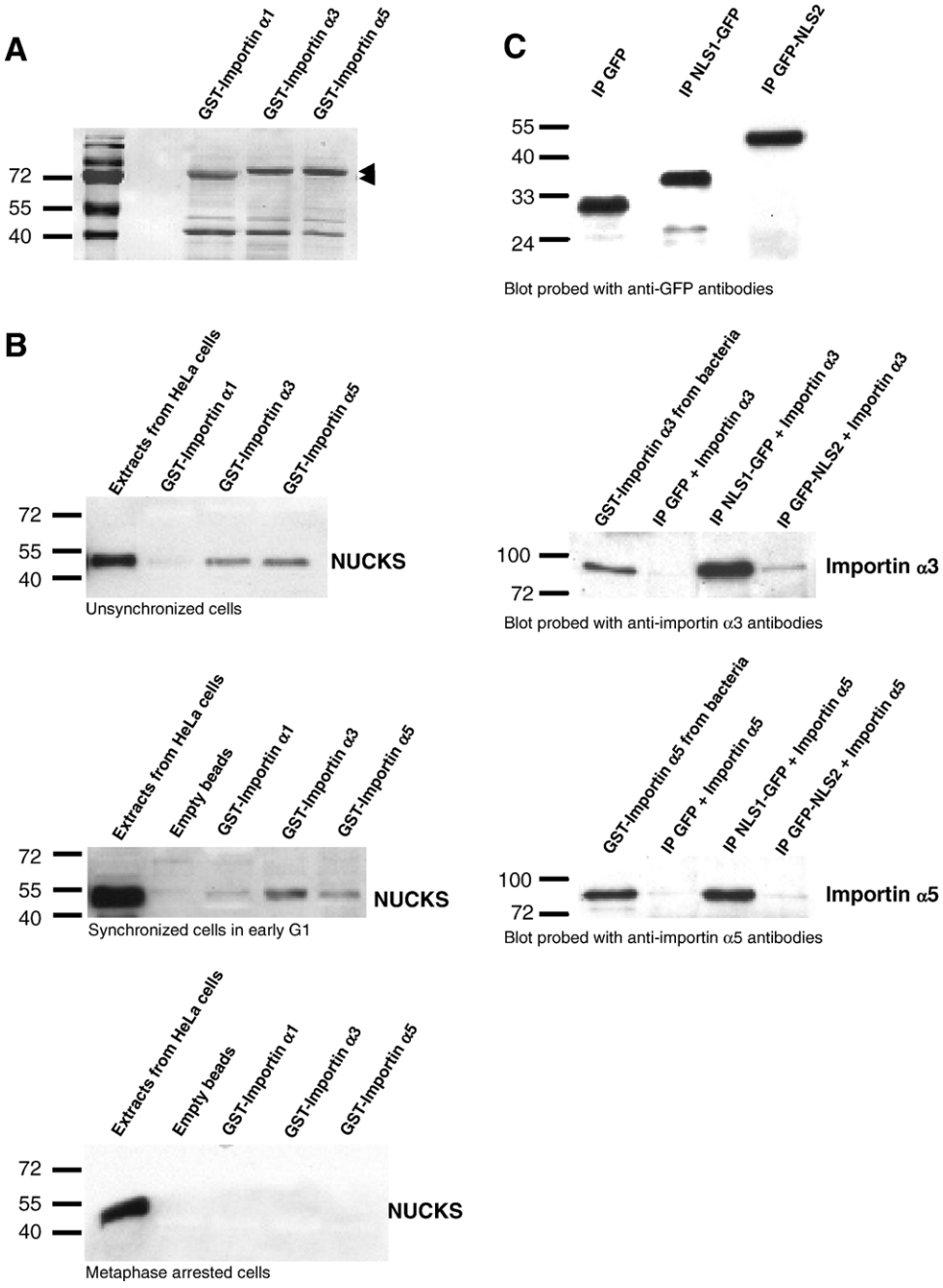

Fig. 5. NUCKS interacts with importin α3 and α5 *in vitro*. (A) Ponceau S stained nitrocellulose form a typical GST-pulldown experiment. The different importins are indicated. (B) GST-importins were immobilized on glutathione–Sepharose beads and incubated with equal amounts of protein extract from unsynchronized HeLa cells, extracts from synchronized HeLa cells (telophase/early G1) and extract from metaphase arrested HeLa cells. Complexes were separated by SDS PAGE and the proteins blotted onto nitrocellulose. The membranes were incubated with affinity purified rabbit anti-NUCKS antibodies followed by a peroxidase-coupled secondary antibody. The blots were then visualized by chemiluminescence. (C) GFP, GFP–NLS1 and GFP–NLS2 were immuno precipitated from lysates of transfected HEK 293 cells with a mouse monoclonal anti-GFP antibody on Protein-G Sepharose beads. After extensive washing, the beads were incubated for 4 h at 4 °C with bacterially expressed GST-importin α molecules. Bound proteins were analysed by Western immunoblotting with polyclonal antibodies directed against GFP, importin α3 and importin α5 followed by a peroxidase-coupled secondary antibody. The blots were then visualized by chemiluminescence.

and hence is among the substrates targeted for nuclear transport in late telophase/early G1, HeLa cells were also synchronized by a double thymidine block, inspected by microscopy and harvested when the majority of the cells were in telophase/early G1. Bound proteins were analysed by Western immunoblotting with anti-NUCKS antibodies. As can be seen from Fig. 5B, NUCKS from unsynchronized cells and thymidine synchronized cells was recognized by importin α3 and α5 but not α1. NUCKS from metaphase arrested cells did not bind to the importin α molecules. To investigate whether each of the two NLSs binds to importin α3 and α5, NLS1–GFP, GFP–NLS2 and GFP (negative control) were immunoprecipitated with a mouse monoclonal anti-GFP antibody. After extensive washing, the immunoprecipitated proteins were incubated with bacterially expressed GST-importin α3 and α5. The bound proteins were analysed by Western immunoblotting with specific polyclonal antibodies against importin α3 and α5. As shown in Fig. 5C, GFP alone did not bind importin α3 or importin α5, while NLS1 bound to both importin subtypes. NLS2 on the other hand, exhibited very low binding to importin α3 (and no binding to importin α5), indicating that the nuclear import of NLS2–GFP observed in Fig. 3 is mediated through another importin subtype.

Altogether, the results indicate that NUCKS interacts with importin α *in vitro*, and that the interaction probably is influenced by metaphase specific, posttranslational modifications of NUCKS.

4. Discussion

Signal-mediated import requires energy, a nuclear localization signal (NLS) and a soluble transport machinery [17]. However, transport studies with model substrates have shown that molecules smaller than 45 kDa can reach the nucleoplasm by diffusion. NUCKS, which has a molecular mass of 27 kDa, should hence be capable of entering the nucleoplasm by free diffusion. We have found that the NUCKS protein is located exclusively in the nuclei of non-cycling cells and proliferating cells in interphase of the cell cycle. This indicates that the nuclear import of NUCKS occurs by an active transport mechanism and not simply by diffusion. NUCKS possesses two potential basic NLSs which may direct it to the nucleus, and we have in this work showed that each of them actively mediates nuclear localization of GFP fusion constructs. In fish, frog and chicken an insert (60 amino acids long), not found in mammals, contains another putative NLS [22]. This potential signal has not been investigated in this work.

The most N-terminal NLS (NLS1) is highly conserved in genetically diverged species and shares perfect homology with the consensus of bipartite NLS sequences present in other karyophilic proteins. Surprisingly, we found that NLS1 in mammals is of monopartite type with the minimal sequence PPTKKIR. The signal resembles the unconventional nuclear signals of RanBP3 (PPVKRERTS) and cMyc, (PAAKRVKLD) [24,29]. In general, very few monopartite NLSs contain as few as three basic amino acids. In NUCKS the absence of a proline N-terminal to the basic core sequence (P32) does not abolish nuclear import, but makes NLS1 less efficient. As a conclusion this proline is important but not essential for the NLS function.

The other putative nuclear localization signal, NLS2, is somewhat less conserved between the species. However, the two basic clusters constituting the bipartite signal are highly conserved, while the spacer region varies. The signal resembles the bipartite signal (NL1) found in the steroid receptor family, $RKX_{10}RKXKK$ [30]. NLS2 directs a truncated form of NUCKS to the nuclei, but the failure of a human splice variant lacking NLS1 to translocate NUCKS to the nuclei, indicates that the main nuclear signal in NUCKS is NLS1. Site directed mutagenesis of the full-length protein confirm these results. The biological significance of NLS2 as a signal for nuclear import is therefore obscure. Interestingly, it has also been reported a splice variant lacking NLS1 in chimpanzee (XM_001162120), implicating a significant biological function for this splicevariant.

It has been shown that cMyc exclusively binds to importin α1 *in vitro* [28], while RanBP3 binds to importin α3 and importin α4 [29]. GST–importin pull down experiments with importin α1, α3 and α5 molecules and extracts from unsynchronized cells or cells harvested in late telophase/early G1 showed that NUCKS exhibited preferential binding to importin α3 and importin α5 *in vitro*. However, all the importin α isoforms failed to bind NUCKS in extracts isolated from cells arrested in metaphase of the cell cycle, indicating that interphase specific posttranslational modifications may stimulate binding to importins while metaphase specific modifications abolish such binding. In conclusion, targeting of NUCKS to the nuclear pore is most likely mediated by a importin α/β heterodimer. The presence of at least one functional NLS and the fact that NUCKS is located in the cell nucleus in proliferating and non-proliferating cells indicate a pivotal role for NUCKS in nuclear functioning.

Acknowledgements

We wish to thank Dr. Toshihiro Sekimoto for his kind gift of vectors containing GST in fusion with importin α1/PTAC 58, importin α3/Qip 1, importin α5/NPI-1 and importin β, Dr. Matthias Köhler for his kind gift of polyclonal antibodies against importin α1, 3 and 5 and Marit Ledsaak for supporting us with the *E. coli* strain. This work was supported by the Norwegian Cancer Society, Anders Jahre's Foundation for the Promotion of Sciences, the Astri and Birger Torstedt legacy, Rakel and Otto Bruuns legacy and the Blix legacy.

References

[1] A.C. Østvold, J.H. Norum, S. Mathiesen, B. Wanvik, I. Sefland, K. Grundt, Molecular cloning of a mammalian nuclear phosphoprotein NUCKS, which serves as a substrate for Cdk1 in vivo, Eur. J. Biochem. 268 (2001) 2430–2440.

[2] A.C. Østvold, U. Haddeland, S.G. Laland, The ubiquity of the highly phosphorylated nuclear protein P1, Biochem. Int. 20 (1990) 991–1000.

[3] T.L. Naylor, J. Greshock, Y. Wang, T. Colligon, Q.C. Yu, V. Clemmer, T.Z. Zaks, B.L. Weber, High resolution genomic analysis of sporadic breast cancer using array-based comparative genomic hybridization, Breast Cancer Res. 7 (2005) 1186–1198.

[4] H.G. Thompson, J.W. Harris, B.J. Wold, S.R. Quake, J.P. Brody, Identification and confirmation of a module of coexpressed genes, Genome Res. 12 (2002) 1517–1522.
[5] H. Shu, S. Chen, Q. Bi, M. Mumby, D.L. Brekken, Identification of phosphoproteins and their phosphorylation sites in the WEHI-231 B lymphoma cell line, Mol. Cell Proteomics 3 (2004) 279–286.
[6] S.A. Beausoleil, M. Jedrychowski, D. Schwartz, J.E. Elias, J. Villen, J. Li, M.A. Cohn, L.C. Cantley, S.P. Gygi, Large-scale characterization of HeLa cell nuclear phosphoproteins, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 12130–12135.
[7] K. Grundt, L. Skjeldal, H.W. Anthonsen, T. Skauge, H.S. Huitfeldt, A.C. Østvold, A putative DNA-binding domain in the NUCKS protein, Arch. Biochem. Biophys. 407 (2002) 168–175.
[8] A.C. Østvold, I. Hullstein, S.G. Laland, The phosphate groups of the high mobility group like protein P1 strengthens its affinity for DNA, Biochem. Biophys. Res. Commun. 185 (1992) 1091–1097.
[9] M. Prymakowska-Bosak, T. Misteli, J.E. Herrera, H. Shirakawa, Y. Birger, S. Garfield, M. Bustin, Mitotic phosphorylation prevents the binding of HMGN proteins to chromatin, Mol. Cell Biol. 21 (2001) 5169–5178.
[10] D.M. Xiao, J.H. Pak, X. Wang, T. Sato, F.L. Huang, H.C. Chen, K.P. Huang, Phosphorylation of HMG-I by protein kinase C attenuates its binding affinity to the promoter regions of protein kinase C gamma and neurogranin/RC3 genes, J. Neurochem. 74 (2000) 392–399.
[11] M.L. Whitfield, G. Sherlock, A.J. Saldanha, J.I. Murray, C.A. Ball, K.E. Alexander, J.C. Matese, C.M. Perou, M.M. Hurt, P.O. Brown, D. Botstein, Identification of genes periodically expressed in the human cell cycle and their expression in tumors, Mol. Biol. Cell 13 (2002) 1977–2000.
[12] D.A. Jans, S. Hubner, Regulation of protein transport to the nucleus: central role of phosphorylation, Physiol. Rev. 76 (1996) 651–685.
[13] M. Breeuwer, D.S. Goldfarb, Facilitated nuclear transport of histone H1 and other small nucleophilic proteins, Cell 60 (1990) 999–1008.
[14] C. Dingwall, R.A. Laskey, Nuclear targeting sequences—A consensus? Trends Biochem. Sci. 16 (1991) 478–481.
[15] D.A. Jans, C.Y. Xiao, M.H. Lam, Nuclear targeting signal recognition: a key control point in nuclear transport? BioEssays 22 (2000) 532–544.
[16] C.A. Hogarth, S. Calanni, D.A. Jans, K.L. Loveland, Importin alpha mRNAs have distinct expression profiles during spermatogenesis, Dev. Dyn. 235 (2006) 253–262.
[17] H. Fried, U. Kutay, Nucleocytoplasmic transport: taking an inventory, Cell. Mol. Life Sci. 60 (2003) 1659–1688.
[18] D. Chelsky, R. Ralph, G. Jonak, Sequence requirements for synthetic peptide-mediated translocation to the nucleus, Mol. Cell Biol. 9 (1989) 2487–2492.
[19] C. Dingwall, J. Robbins, S.M. Dilworth, B. Roberts, W.D. Richardson, The nucleoplasmin nuclear location sequence is larger and more complex than that of SV-40 large T antigen, J. Cell Biol. 107 (1988) 841–849.
[20] S. Chatterjee, U. Stochaj, Monitoring nuclear transport in HeLa cells using the green fluorescent protein, Biotechniques 21 (1996) 62–63.
[21] P. Volpe, T. Eremenko, A method for measuring cell cycle phases in suspension cultures, Methods Cell Biol. 6 (1973) 113–126.
[22] K. Grundt, I.V. Haga, V. Aleporou-Marinou, Y. Drosos, B. Wanvik, A.C. Østvold, Characterisation of the NUCKS gene on human chromosome 1q32.1 and the presence of a homologous gene in different species, Biochem. Biophys. Res. Commun. 323 (2004) 796–801.
[23] D. Christophe, C. Christophe-Hobertus, B. Pichon, Nuclear targeting of proteins: how many different signals? Cell Signal. 12 (2000) 337–341.
[24] J.P. Makkerh, C. Dingwall, R.A. Laskey, Comparative mutagenesis of nuclear localization signals reveals the importance of neutral and acidic amino acids, Curr. Biol. 6 (1996) 1025–1027.
[25] P. Cui, B. Qin, N. Liu, G. Pan, D. Pei, Nuclear localization of the phosphatidylserine receptor protein via multiple nuclear localization signals, Exp. Cell Res. 293 (2004) 154–163.
[26] D. Gorlich, U. Kutay, Transport between the cell nucleus and the cytoplasm, Annu. Rev. Cell Dev. Biol. 15 (1999) 607–660.
[27] M. Kohler, S. Ansieau, S. Prehn, A. Leutz, H. Haller, E. Hartmann, Cloning of two novel human importin-alpha subunits and analysis of the expression pattern of the importin-alpha protein family, FEBS Lett. 417 (1997) 104–108.
[28] S.G. Nadler, D. Tritschler, O.K. Haffar, J. Blake, A.G. Bruce, J.S. Cleaveland, Differential expression and sequence-specific interaction of karyopherin alpha with nuclear localization sequences, J. Biol. Chem. 272 (1997) 4310–4315.
[29] K. Welch, J. Franke, M. Kohler, I.G. Macara, RanBP3 contains an unusual nuclear localization signal that is imported preferentially by importin-alpha3, Mol. Cell Biol. 19 (1999) 8400–8411.
[30] D. Picard, K.R. Yamamoto, Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor, EMBO J. 6 (1987) 3333–3340.